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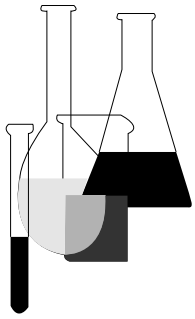
Health Effects Test Guidelines

OPPTS 870.8800 Morphologic Transformation of Cells in Culture



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

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OPPTS 870.8800 Morphologic transformation of cells in culture.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is OPPT 40 CFR 795.285 Morphologic Transformation of Cells in Culture.

(b) **Purpose.** In vitro assays for cellular transformation are semiquantitative assays for the ability of chemical agents to transform (morphologically alter) cells in culture. Such transformation is associated with certain phenotypic changes such as loss of contact inhibition and the ability to form colonies in soft agar medium. The process by which these changes occur is assumed to be closely related to the process of in vivo carcinogenesis. Morphologically transformed cells appear as foci of dense, piled-up, altered cells on an underlying monolayer of normal cells. Three types of foci have been recognized. Type III foci appear to be most closely correlated with in vivo tumor formation. The ultimate criterion for morphologic transformation is the ability of the transformed cells to induce tumors when inoculated into appropriate hosts. Not all cells which appear to be morphologically transformed are capable of tumor formation. In general, there is reasonably good correlation between in vitro transformation and in vivo oncogenesis, although the correlation varies depending on the system being studied. These systems are believed to be reasonably good predictors of in vivo activity, and positive results are viewed as potential indications of in vivo carcinogenesis.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Morphologic transformation is the acquisition of certain phenotypic characteristics, most notably loss of contact inhibition and loss of anchorage dependence, which are often but not always associated with the ability to induce tumors in appropriate hosts.

Type III foci of transformed cells are multilayered aggregations of densely staining cells with random orientation and criss-cross arrays at the periphery of the aggregate. They appear as dark stained areas on a light staining background monolayer which is one-cell thick.

(d) **Test method**—(1) **Principle.** (i) Three systems for detecting chemically induced morphologic transformation have been described—systems which employ cell lines (cells with an indefinite lifespan), systems which employ cell strains (cells with a finite or limited lifespan), and systems which detect the interaction between chemicals and oncogenic viruses.

(ii) This study should employ an established cell line for detection of morphologic transformaton.

(2) **Description.** Cells in culture are exposed to the test substance, both with and without metabolic activation, for a defined period of time. Cytotoxicity is determined by measuring the colony-forming ability and growth rate of the cultures after the treatment period. At the end of the treatment period, cultures are maintained in growth medium for a sufficient period of time to allow near-optimal expression of transformed foci.

(3) **Cells.** (i) Balb/c–3T3 mouse cells originally obtained from clone A–31 or its derivatives should be used in the assay. Cells should be checked for mycoplasm contamination prior to use in the assay and may be checked for karyotype.

(ii) Appropriate culture media and incubation conditions (culture vessels, CO_2 concentrations, temperature, and humidity) should be used.

(4) **Metabolic activation.** Cells should be exposed to test substance both in the presence and absence of a metabolic activation system. The metabolic activation system should be derived from primary cultures of rat hepatocytes.

(5) **Control groups.** Positive and negative (untreated and vehicle) controls should be included in each experiment. 3-Methylcholanthrene is an example of a positive control for experiments without metabolic activation. Dimethylnitrosamine is an example of a positive control in experiments with metabolic activation.

(6) **Test chemicals**—(i) **Vehicle.** Test agents should be dissolved in serum-complete culture medium prior to treatment of the cells.

(ii) **Exposure concentrations.** Several concentrations (usually at least four) of the test substance should be used. These should be selected on the basis of a preliminary cytotoxicity assay performed both with and without metabolic activation. The highest concentration should produce a low level of survival (approximately 10 to 20 percent), and the survival in the lowest concentration should approximate that of the negative control.

(e) **Test performance.** (1) Cells should be exposed to the test substance both with and without metabolic activation. Exposure should be for 72 h for experiments without metabolic activation and for 48 h for experiments with metabolic activation unless different exposure times are justified by the investigator.

(2) At the end of the exposure period, cells should be washed and cultured to determine viability and to allow for expression of transformation.

(3) At the end of the incubation period (generally 4 to 6 weeks), cells should be fixed and stained, and the number of transformed (Type III) foci should be enumerated.

(4) All results should be confirmed in an independent experiment if a single, statistically significant positive effect is produced at one dose point without a dose response. A positive response should be confirmed by testing over a narrow range of concentrations.

(5) Tumorigenic potential of isolated morphologically transformed foci may be determined by inoculation into suitable hosts.

(f) **Data and report**—(1) **Treatment of results.** (i) Data should be presented in tabular form. Individual colony counts for the treated and control groups should be presented for both transformation and survival.

(ii) Survival and cloning efficiencies should be given as a percentage of the controls. Transformation should be expressed as a number of foci per dish, the number of dishes with transformed foci, and the number of transformed foci per number of surviving cells.

(2) **Interpretation of results.** (i) There are several criteria for determining a positive result, one of which is a statistically significant concentration-related increase in the number of transformed foci. Another criterion may be based upon the detection of a reproducible and statistically significant positive response for at least one of the test substance concentrations.

(ii) A test substance which does not produce either a statistically significant concentration-related increase in the number of transformed foci or a statistically significant and reproducible positive response at any one of the test points is considered to be negative in this system.

(iii) Both biological and statistical significance should be considered together in the evaluation.

(3) **Test evaluation.** (i) Positive results for an in vitro mammalian cell transformation assay indicate that, under the test conditions, the test substance induces morphologic transformation in the cultured mammalian cells used.

(ii) Negative results indicate that, under the test conditions, the test substance does not induce morphologic transformation in the cultured mammalian cells used.

(4) **Test report.** In addition to the reporting recommendations as specified under 40 CFR part 792, subpart J, the following specific information should be reported:

(i) Cell type used, including subclone designation and passage number; number of cell cultures; methods used for maintenance of cell cultures.

(ii) Rationale for selection of concentrations and number of cultures.

(iii) Test conditions: Composition of media, CO_2 concentration, concentration of test substance, vehicle, incubation temperature, incubation time, duration of treatment, cell density during treatment, type of metabolic activation system, positive and negative controls, length of expression period (including number of cells seeded and subculture and feeding schedules, if appropriate).

(iv) Methods used to enumerate numbers of viable cells and transformed foci.

(v) Dose-response relationship, where possible.

(g) **References.** The following references should be consulted for additional background information on this test guideline.

(1) Heidelberger, C. et al. Cell transformation by chemical agents—a review and analysis of the literature: a report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutation Research* 114:283–385 (1983).

(2) Kakunaga, T. A quantitative system for assay of malignant transformation by carcinogens using a clone derived from Balb-3T3. *International Journal of Cancer* 12:463–473 (1973).

(3) Reznikoff, C.A. et al. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Research* 33:3239–3249 (1973).

(4) Reznikoff, C.A. et al. Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. *Cancer Research* 33:3231–3238 (1973).

(5) Sivak, A. et al. Balb/c–3T3 cells as target cells for chemically induced neoplastic transformation. In: *Advances in modern environmental toxicology, mammalian cell transformation by chemical carcinogens,* Vol. I. Mishra, N., Dunkel, V., Mehlman, M., eds. Senate Press, Princeton Junction, NJ. pp. 133–180 (1981).

(6) Sivak, A. and Tu, A.S. Factors influencing neoplastic transformation by chemical carcinogens in Balb/c-3T3 cells. In: *The predictive value of short-term screening tests in carcinogenicity evaluation.* Williams, G.M., Kroes, R., Waaijers, H.W., Van de Poll, K.W., eds. Elsevier/North

Holland Biomedical Press, Amsterdam, New York, Oxford pp. 177–190 (1980).

(7) Williams, G.M. Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Research* 37:1845–1851, (1977).